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Synthesis, pharmacokinetics and anticonvulsant activity of 7-chlorokynurenic acid prodrugs

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Abstract

7-Chlorokynurenic acid **1** is a potent glycine-*N*-methyl-D-aspartate (NMDA) receptor antagonist, but it shows weak activity after systemic administration. In order to overcome the Blood–brain barrier (BBB), we synthetized three new esters **2**–**4** of **1** obtained by chemical conjugation with essential nutrients such as glucose and galactose, that are actively transported across the BBB. These compounds were assayed to evaluate their in vitro chemical and enzymatic hydrolysis. In addition the prodrugs **2**–**4** were tested for their ability to protect mice against NMDA-induced seizures after systemic administration. All the prodrugs **2**–**4** appeared moderately stable in pH 7.4 buffered solution and were susceptible to in vitro enzymatic hydrolysis. Intraperitoneal administration of either esters **2** or **4** was highly protective against seizures induced by NMDA in mice, with the latter prodrug showing the highest anticonvulsive activity. In addition, ester **4** undergoes a time-dependent extracellular hydrolysis into **1** when applied to mixed cultures of mouse cortical cells, a model that reproduces in vitro the cellular milieu encountered by the prodrugs once they penetrate the brain parenchyma.

Keywords: Prodrug; 7-Chlorokynurenic acid; Blood–brain barrier; NMDA-receptor; Anticonvulsant

1. Introduction

The blood–brain barrier (BBB) restricts the brain uptake of many valuable hydrophilic drugs and limits their efficacy in the treatment of brain diseases. In fact the brain microvessel endothelium provides a barrier to the passive transport of

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hydrophilic drugs into the brain. Several strategies have been developed to overcome this problem (Greenwood, 1992; Greig, 1992; Robinson and Rapoport, 1992). The prodrug approach represents a very promising method to enhance drug delivery to the brain. Prodrugs are normally inactive and must generate the active drug at their target by enzymatically or chemically mediated cleavage of their promoiety. The development of CNS-active prodrugs has been generally aimed at obtaining an improvement in their lipophilic character, by masking transiently ionized groups of the parent drug.

However, higher lipophilic character can be a limit for the prodrug entering the brain owing to plasma binding or rapid peripheral distribution and elimination: the benefits achieved in barrier permeability by lipophilicity enhancement are, in some instances, offset by changes in plasma protein binding and/or peripheral distribution and elimination, that reduce the concentration of free drug available for brain transport.

Otherwise, the preferential delivery of prodrugs to the brain may be improved by using endogenous facilitated transport systems, present at the blood–brain interface. Thus chemical conjugation of potentially CNS-active drugs with an aminoacidic or glycoside moiety actively transported across the BBB, represents a plausible means of improving their brain delivery, by providing suitable substrates for active membrane transport. In particular, conjugation with tyrosine or glucose has recently been shown to be a successful means of selective drug delivery; for example, phosphonoformate-L-tyrosine conjugate is actively transported (Walker et al., 1994), by means of active amino acid carriers, through monolayers of porcine brain microvessel endothelial cells, and a glycosyl phosphotriester prodrug of 3′-azido-3′-deoxythymidine (AZT) shows a good delivery to the CNS (Namane et al., 1992). Similarly, conjugating glucose or galactose to poorly absorbable drugs can improve their intestinal absorption by means of glucose transport carriers in the small intestine (Mizuma et al., 1992, 1993, 1994), i.e. tocopherol conjugation to a monocarboxylate or glycoside moiety has appeared to provide suitable substrates for active erythrocyte membrane transport (Bonina et al., 1996).

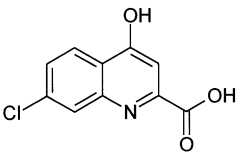

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Fig. 1. Structure of 7-Cl-Kynurenic acid.

In addition, in our previous paper (Bonina et al., 1999), this strategy was already applied with success to the synthesis of new prodrugs of nipecotic acid, a potent in vitro inhibitor of GABA uptake, which is inactive as anticonvulsant when administered systematically. These nipecotic acid esters were obtained by chemical conjugation with essential nutrients, such as glucose, galactose or tyrosine, that are actively transported across the BBB.

7-chlorokynurenic acid **1** (Fig. 1) is one of the most potent antagonists at the glycine site of the *N*-Methyl-D-aspartate (NMDA) receptor (Kemp et al., 1988; Kemp and Leeson, 1993) and is therefore of potential therapeutic interest as a neuroprotective agent. However, the therapeutic use of this compound in acute or chronic neurodegenerative disorders is limited by its low CNS availability which may be related to inefficient transport across the BBB: it is therefore inactive when administered systematically (Kemp and Leeson, 1993). The purpose of this work was to synthesize new esters **2**–**4** of 7-chlorokynurenic acid obtained by chemical conjugation with BBB actively-transported essential nutrients, such as glucose and galactose. These compounds **2**–**4** were assayed to evaluate their 'in vitro' chemical and enzymatic hydrolysis. Besides these, new esters **2**–**4** were tested to evaluate their ability to protect against NMDA-induced seizures in mice after systemic administration. In addition, to examine whether compounds **2**–**4** were able to release the parent drug **1** at the target site, we evaluated the enzymatic cleavage of these esters in mixed cultures of neurones and glial cells, a model that roughly reproduces the cellular milieu encountered by a CNS-prodrug after it crosses the BBB.

2. Methods and materials

2.1. Materials

All other reagent chemicals were obtained from Aldrich Chemical Company. LC grade acetonitrile and water, used in HPLC, were obtained from Merck and Carlo Erba, respectively.

2.2. Apparatus

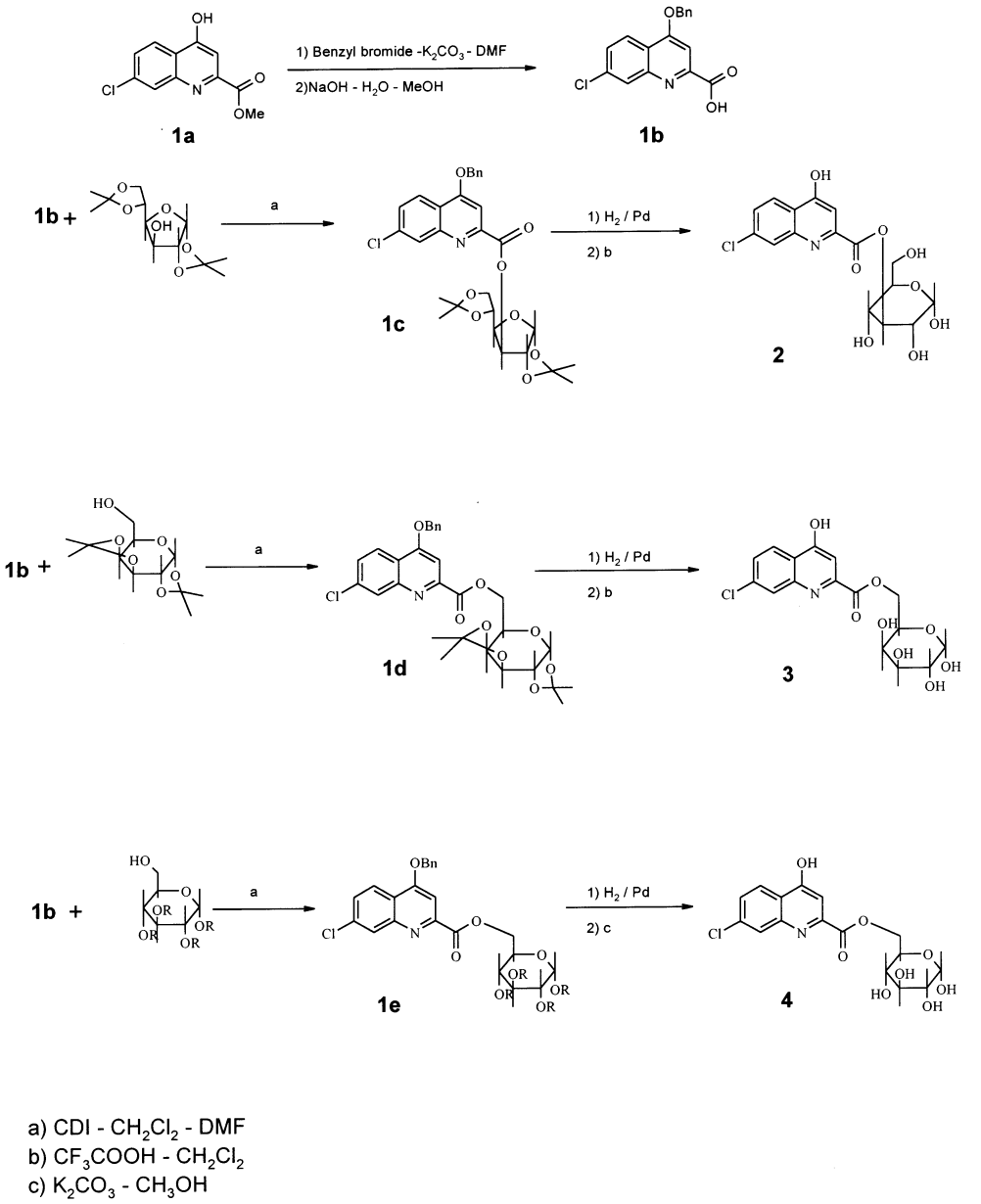
Proton and carbon-13 nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AMX-500 spectrometer equipped with a Bruker X-32 computer. For spectra measured in organic solvents, data are reported in ppm from internal tetramethylsilane for ^{1}H NMR and in ppm from the solvent in ^{13}C NMR. Data are reported as follows: chemical shift; multiplicity (s, singlet; d , doublet, m, multiplet); coupling constant; and integration. Infrared (IR) spectra were measured on a Bruker FT-IR IFS-48. Melting points (mp) were determined with a Kofler hot stage microscope and are uncorrected. Elemental analyses indicated by the symbols of the elements were performed on a Perkin–Elmer model 240 elemental analyzer, and were within $\pm 0.4\%$ of theoretical values. Flash chromatography was performed on Merck silica gel (0.040–0.063 mm). Reverse phase chromatography was performed on Aldrich octadecyl-functionalized silica gel. High performance liquid chromatography (HPLC) analyses were performed on Beckman Instrument Inc. Fullerton CA, a C-18 column (Ultrasphere ODS 3μm Spherical, 80 A pore, 4.6×75 mm; Beckman), and a RF-551 spectrofluorimetric detector (Shimadzu).

2.3. General synthetic method

The three ester prodrugs **2**, **3** and **4** were synthesised from 7-chlorokynurenic acid methyl ester (**1a**), obtained using a synthetic method reported in literature (Surrey and Hammer, 1946a,b; Scheme 1). Reaction of **1a** with benzyl bromide in dimethylformamide in the presence of K_2CO_3 followed by hydrolysis gave the 4-benzyloxy-2-carboxylic acid **1b**. Esterification of **1b** with 1, 2:4, 5-di-*O*-isopropylidene-α-D-glucofuranose, 1, 2:3, 4-di-*O*-isopropylidene-α-D-galactopyranose and 1, 2, 3, 4-*O*-tetramethylcarbonate-D-glucopyranose, in the presence of 1,1[1]-carbonyldiimidazole (CDI), afforded **1c** (68%), **1d** (75%) and **1e** (65%), respectively. The benzyl group was cleaved by hydrogenolysis and the protecting groups of the resultant esters were removed with trifluoroacetic acid (TFA) in CH_2Cl_2 followed by purification on octadecyl-functionalized silica gel to afford **2** and **3** to yield 85 and 87%, respectively. On the other hand the compound **1e** was cleaved with K_2CO_3 3% in methyl alcohol and then purified on octodecyl-functionalized silica gel to give **4** (80%). The 1,2:4,5-di-*O*-isopropylidene-α-D-glucofuranose and 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose are commercially available, while the 1,2,3,4-*O*-tetramethylcarbonate-D-glucose was synthesized in our laboratory.

2.3.1. 4-Benzyloxy-7-chlorokynurenic acid (1b)

7-Chlorokynurenic acid methyl ester **1a**, (4 g, 16.87 mmol) and K_2CO_3 (2.79 g, 20.24 mmol) were dissolved in DMF (60 ml). Benzyl bromide (2.4 ml, 3.46 g, 20.24 mmol) was added over a period of 15 min and the reaction mixture was heated at reflux. After 4 h solvents were removed by vacuum. The residue was dissolved in H_2O-CH_3OH (100 ml, 1:1) and NaOH (4 g, 100 mmol) was added. After stirring at room temperature for 2 h, the methanol was removed and the resulting aqueous solution was acidified (to pH 4–5) with 6 M HCl and then extracted with ethyl acetate (3×60 ml). The combined organic phases were dried (Na_2SO_4) and evaporated, after flash filtration of the residue on silica gel afforded the title compound as a yellow solid (4.65 g, 88%); mp 135–136°C; ^{1}H NMR ($CDCl_3$) $\delta = 8.07$ (d, $J = 11$ Hz, 1H, H-6), 7.92 (s, 1H, H-8), 7.40–7.18 (m, 7H, H-3, H-5 and phenyl group), 5.22 (s, 2H, $PhCH_2$); ^{13}C NMR ($CDCl_3$) $\delta =$ 170.36, 161.79, 148.03, 142.39, 138.14, 136.89, 131.38, 130.08, 129.83, 129.41, 128.91, 126.09, 121.98, 104.01, 68.66; Anal. ($C_{17}H_{12}NO_3Cl$) C, H, N.



Scheme 1. General synthetic method.

*2.3.2. 4-Benzyloxy-7-chlorokynurenic acid (1′, 2′:5′, 6′-diisopropylidene)-α-D-glucofuranos-3′-yl ester (**1c**).*

1:1[1]-Carbonyldiimidazole (0.648 g, 4 mmol) was added to a stirred solution of 4-benzyloxy-7-chlorokynurenic acid **1b** (1.2 g, 3.83 mmol) in dry CH_2Cl_2-DMF (50 ml, 7:3). After 2 h, 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (1.2 g, 4.61 mmol) in dry CH_2Cl_2 (20 ml) was added dropwise to this mixture over 20 min and stirring continued for 12 h. Evaporation of the solvent gave a residue which was taken up in ethyl acetate (50

ml) and washed with water (2 × 15 ml). The organic layer was dried (Na_2SO_4) and evaporated to give a residue which was submitted to flash chromatography, eluting with petroleum ether-ethyl acetate (9:1) to afford the title compound as a pale yellow oil (1.45 g, 68%); ^{1}H NMR ($CDCl_3$) δ = 8.05 (d, J = 11 Hz, 1 H, H-6), 7.97 (s, 1H, H-8), 7.40–7.18 (m, 7H, H-3, H-5 and phenyl group), 5.88–5.80 (m, 1H, H-1^1), 5.40–5.32 (m, 1H, H-3^1), 5.15 (s, 2H, $PhCH_2$), 4.55–4.45 (m, 1H, H-2^1), 4.28–4.16 (m, 2H, H-4^1 and H-5^1), 4.10–3.90 (m, 2H, H-6^1), 1.50 + 1.40 + 1.22 + 1.19 + 1.09 (s, 12H, ketals); ^{13}C NMR ($CDCl_3$) δ = 171.36, 162.88, 146.99, 142.60, 138.00, 136.03, 130.42, 130.04, 129.89, 128.29, 128.20, 127.06, 120.99, 111.56, 109.41, 108.05, 105.00, 84.77, 80.78, 74.95, 73.23, 68.32, 67.37, 26.54, 25.89, 24.84; Anal. ($C_{29}H_{30}NO_8Cl$) C, H, N.

*2.3.3. 4-Benzyloxy-7-chlorokynurenic acid-(1′, 2′:3′, 4′-di-isopropylidene)-α-D-galactopyranos-6′-yl ester (**1d**)*

The ester 1d (1.2 g, 75%) was prepared as a pale yellow oil from of 4-benzyloxy-7-chlorokynurenic acid **1b** (0.9 g, 2.87 mmol), and 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose (0.9 g, 3.45 mmol) in the presence of 1,1^1-carbonyl-di-imidazole (0.485 g, 2.99 mmol) by the procedure described previously for making the compound **1c**; ^{1}H NMR ($CDCl_3$) δ = 7.90(s, 1 H, H-8), 7.80 (d, J = 11 Hz, 1H, H-6), 7.18–7.00 (m, 7H, H-3, H-5 and Phenyl group), 5.25-5.20 (m, 1H, H-1^1), 4.93 (s, 2H, $PhCH_2$), 4.35-4.25 (m, 3H, H-2^1, H-3^1 and H-5^1), 4.05–4.00 (m, 2H, H-6^1), 3.98–3.90(m, 1H, H-4^1), 1.25 + 1.15 + 1.05 + 1.00 (s, 12H, ketals); ^{13}C NMR ($CDCl_3$) 171.88, 163.32, 144.05, 142.78, 138.21, 136.22, 130.15, 129.36, 127.88, 127.31, 126.99, 126.31, 122.34, 111.31, 109.12, 108.37, 95.97, 71.16, 70.40, 70.23, 68.21, 67.89, 61.82, 25.68, 24.62, 23.98; Anal. ($C_{29}H_{30}NO_8Cl$) C, H, N.

*2.3.4. 4-Benzyloxy-7-chloro-kynurenic acid (1′, 2′, 3′, 4′-O-tetramethylcarbonate)-D-glucopyranos-6′-yl ester (**1e**)*

The ester **1e** (1.47 g, 65%) was prepared as a pale yellow oil from 4-benzyloxy-7-chlorokynurenic acid **1b** (1 g, 3.19 mmol) and 1, 2, 3, 4-tetramethylcarbonate-D-glucose (1.58 g, 3.83 mmol) in the presence of 1,1^1-carbonyl-di-imidazole (0.538 g, 3.32 mmol) by the procedure described previously for making compound **7**. The desired product was purified by flash chromatography using petroleum ether-ethyl acetate (1:1); ^{1}H NMR ($CDCl_3$) δ = 8.00 (d, J = 11 Hz, 1 H, H-6), 7.87 (s, 1H, H-8), 7.35–7.18 (m, 7H, H-3, H-5 and phenyl group), 5.50–5.40 (m, 1H, H-1^1), 5.20–5.15 (m, 2H, $PhCH_2$), 4.50–4.40 (m, 1H, H-3^1), 4.10–3.95 (m, 1H, H-2^1), 3.90–3.80 (m, 2H, H-4^1 and H-5^1), 3.60–3.40 (m, 14H, H-6^1 and CO_2CH_3); ^{13}C NMR ($CDCl_3$) δ = 171.55, 162.56, 153.31, 153.20, 153.10, 144.31, 140.56, 139.99, 139.79, 138.42, 136.50, 136.31, 131.42, 130.60, 129.42, 129.30, 129.14, 128.99, 128.62, 128.55, 119.55, 119.21, 110.40, 109.98, 100.51, 96.03, 80.51, 80.30, 77.15, 75.20, 74.88, 73.41, 68.81, 67.70, 53.61, 53.44, 53.10, 52.44, 52.13, 51.99, 51.80; Anal. ($C_{31}H_{30}NO_{16}Cl$) C, H, N.

2.3.5. 7-Chlorokynurenic acid-(1′, 2′:5′, 6′-di-isopropylidene)-α-D-glucofuranos-3′-yl ester

Hydrogen was bubbled for 10 min into a magnetically stirred suspension of ester **1c** (1.2 g, 2.16 mmol) in CH_3OH (46 ml) containing 10% Pd-C(0.12 g) at room temperature. Filtration of the mixture through Celite and evaporation gave a residue which was submitted upon flash chromatography on silica gel; elution with $CHCl_3$-CH_3OH 95:5 afforded the title compound a white solid (0.954 g, 95%); mp 175–176°C; ^{1}H NMR (MeOD) δ = 8.23 (d, J = 11 Hz, 1H, H-6), 7.87 (s, 1H, H-8), 7.42 (d, J = 11 Hz, 1H, H-5), 6.98 (s, 1H, H-3), 5.86–5.80 (m, 1H, H-1^1), 5.38–5.30 (m, 1H, H-3^1), 4.52–4.43 (m, 1H, H-2^1), 4.18–4.10 (m, 2H, H-4^1 and H-5^1), 4.08-3.90 (m, 2H, H-6^1), 1.45 + 1.40 + 1.27 + 1.10 (s, 12H, ketals); ^{13}C NMR (MeOD) δ = 179.92, 163.26, 147.12, 142.53, 136.92, 128.31, 126.93, 124.47, 121.68, 111.21, 110.31, 109.30, 104.83, 84.71, 80.78, 75.01, 73.50, 67.61, 26.51, 25.31, 24.94; Anal. ($C_{22}H_{24}NO_8Cl$) C, H, N.

2.3.6. 7-Chlorokynurenic acid-(1′, 2′:3′, 4′-di-isopropylidene)-α-D-galactopyranos-6′-yl ester

With the procedure previously described, the ester **1d** (1.3g, 2.34 mmol) was converted to the

title compound (1.05 g, 96%) as a white solid; mp 168–169°C; ^{1} H NMR (MeOD) $\delta = 8.21$ (d, $J =$ 11 Hz, 1H, H-6), 7.90 (s, 1H, H-8), 7.41 (d, $J = 11$ Hz, 1H, H-5), 7.00 (s, 1H, H-3), 5.50–5.42 (m, 1H, H-1^1), 4.33–4.25 (m, 3H, H-2^1, H-3^1 and H-5^1), 4.10–4.03 (m, 2H, H-6^1), 3.97–3.88(m, 1H, H-4^1), $1.20 + 1.12 + 1.05 + 0.99$ (s, 12H, ketals); ^{13}C NMR (MeOD) $\delta = 178.00$, 162.73, 145.95, 142.88, 136.35, 128.15, 125.92, 124.15, 122.03, 109.31, 109.12, 108.21, 95.66, 71.00, 70.40, 70.10, 67.61, 62.01, 25.31, 24.80, 24.08; Anal. ($C_{22}H_{24}NO_8Cl$) C, H, N.

2.3.7. 7-Chlorokynurenic acid (1′, 2′, 3′, 4′,-O-tetramethylcarbonate)-D-glucopyranos-6′-yl ester

The ester **1e** (1 g, 1.41 mmol) was hydrogenated as described above to yield 0.81 g (93%) of the title compound as a pale yellow oil; ^{1}H NMR (MeOD) $\delta = 8.25$ (d, $J = 11$ Hz, 1H, H-6), 7.90 (s, 1H, H-8), 7.42 (d, $J = 11$ Hz, 1H, H-5), 7.06 (s, 1H, H-3), 5.12-5.05 (m, 1H, H-1^1), 4.40–4.35 (m, 1H, H-3^1), 4.10–3.98 (m, 1H, H-2^1), 3.92–3.85 (m, 2H, H-4^1 and H-5^1), 3.62–3.39 (m, 14H, H-6^1 and CO_2CH_3); ^{13}C NMR (MeOD) $\delta = 179.51$, 179.15, 162.61, 154.88, 143.16, 140.56, 140.31, 136.86, 128.53, 125.99, 125.61, 124.10, 120.35, 119.73, 110.32, 109.71, 99.46, 95.31, 79.43, 77.23, 76.41, 75.52, 74.76, 73.33, 70.78, 65.15, 54.16, 53.88, 53.60, 53.40, 53.10, 52. 89, 52.93; Anal ($C_{24}H_{24}NO_{16}Cl$) C, H, N.

2.3.8. 7-Chlorokynurenic acid D-glucos-3′-yl ester (2)

Trifluoroacetic acid (5 ml, 65.29 mmol) was added to a solution of debenzylated-1c (0.5 g, 1 mmol) in CH_2Cl_2 (5 ml) and the mixture was stirred for 24 h at room temperature. Evaporation of the solvent gave a residue which was submitted to chromatography on octodecyl-functionalized silica gel, eluting with H_2O-CH_3OH 1:1, to give the title compound as a white solid (0.327 g, 85%); mp 119–120°C; ^{1}H NMR (MeOD) $\delta = 8.23$ (d, $J = 11$ Hz, 1H, H-6), $7.93 + 7.82$ (s, 1H, H-8, two anomers), 7.48 (d, $J = 11$ Hz, 1H, H-5), $7.08 + 6.93$ (s, 1H, H-3, two anomers), 5.63-5.48 (m, 1H, H-1^1), 5.30–5.26 (m, 1H, H-3^1), 4.06–4.00 (m, 1H, H-2^1), 3.93–3.65 (m, 4H, H-4^1, H-5^1 and H-6^1); ^{13}C NMR (MeOD) $\delta = 180.72$, 162.96, 143.70, 142.50, 142.10, 136.47, 128.39, 128.08, 126.52, 126.02, 120.10, 119.46, 112.35, 111.89, 98.17, 93.85, 82.16, 80.30, 77.66, 76.22, 73.75, 62.79, 62.68; IR (KBr) 3650–3200 (br), 1737, 1715; Anal. ($C_{16}H_{16}NO_8Cl$) C, H, N.

2.3.9. 7-Chlorokynurenic acid D-galactos-6′-yl ester (3)

A solution of debenzylated-**1d** (0.7 g, 1.5 mmol) and trifluoroacetic acid (7 ml, 91.40 mmol) in CH_2Cl_2 (7 ml) was stirred for 26 h at room temperature. Evaporation of the solvent produced a residue which was submitted to chromatography on octodecyl-functionalized silica gel eluting with H_2O-CH_3OH 1:1, to give 0.5 g (87%) of the pure **3** as a white solid; mp 129–130°C; ^{1}H NMR (MeOD) $\delta = 8.24$ (d, $J = 11$ Hz, 1H, H-6), $7.95 +$ 7.80 (s, 1H, H-8, two anomers), 7.48 (d, $J = 11$ Hz, 1H, H-5), $7.00 + 6.92$ (s, 1H, H-3, two anomers), 5.23–5.20 (m, 1H, H-1^1), 4.80–4.60 (m, 3H, H-2^1, H-3^1 and H-5^1), 4.05–3.95 (m, 2H, H-6^1), 3.85–3.75 (m, 1H, H-4^1); ^{13}C NMR (MeOD) $\delta = 181.02$, 161.55, 142.42, 141.58, 141.10, 136.35, 128.44, 128.12, 126.22, 124.11, 120.06, 118.99, 112.31, 110.99, 98.21, 93.83, 79.21, 78.44, 77.53, 76.31, 72.99, 66.83, 66.63; IR (KBr) 3550–3150 (br), 1740, 1724; Anal. ($C_{16}H_{16}NO_8Cl$) C, H, N.

2.3.10. 7-Chlorokynurenic acid D-glucos-6′-yl ester (4)

A solution of K_2CO_3 (0.3 g, 3 mmol) in methanol (30 ml) was added to a solution of debenzylated-**1e** (0.8 g, 1.29 mmol) in CH_3OH (10 mL) and the mixture was stirred for 30 h at room temperature. Evaporation of the solvent produced a residue which was submitted to chromatography on octodecyl-functionalized silica gel eluting with H_2O-CH_3OH 1:1, to give the title compound as a white solid (0.4 g, 80%); mp 115–116°C. ^{1}H NMR (MeOD) $\delta = 8.20$ (d, $J = 11$ Hz, 1H, H-6), $7.90 + 7.81$ (s, 1H, H-8, two anomers), 7.45 (d, $J = 11$ Hz, 1H, H-5), $7.06 + 6.90$ (s, 1H, H-3, two anomers), 5.30–5.18 (m, 1H, H-1^1), 5.07–5.02 (m, 1H, H-3^1), 4.10–4.07 (m, 1H, H-2^1), 3.90–3.63 (m, 4H, H-4^1, H-5^1 and H-6^1). ^{13}C NMR (MeOD)

$\delta = 179.43$, 179.06,162.32, 140.27, 140.00, 136.98, 128.88, 128.78, 125.25, 125.10, 120.05, 119.33, 110.51, 109.38, 97.36, 92.79, 79.46, 76.93, 75.35, 73.54, 72.41, 69.49, 67.16; IR (KBr) 3700–3350 (br), 1735, 1722; Anal. ($C_{16}H_{16}NO_8Cl$) C, H, N.

2.4. Chemical stability

7-Chlorokynurenic ester solutions were prepared by dissolving an aliquot of compounds (**2**–**4**) in pH 7.4 phosphate buffer to give a final concentration of about 10^{-5} M. The solution was maintained at 37°C and aliquots were withdrawn every 1 h for the initial 12 h of incubation. The disappearance of the 7-chlorokynurenic esters was followed by HPLC analysis using the method reported below. Pseudo-first-order rate constants for chemical hydrolysis were determined from the slopes of linear plots obtained by reporting the logarithm of residual nipecotic ester against time. All experiments were carried out in triplicate.

2.5. Enzymatic stability

Enzymatic hydrolysis of 7-chlorokynurenic esters (**2**–**4**) was determined using the procedure described in the literature (Bonina et al. 1993).

Porcine liver esterase (Sigma) was diluted 10 times with phosphate buffer and used to hydrolyze 7-chlorokynurenic esters. Ester solutions were prepared by dissolving an aliquot of compounds **2**–**4** in phosphate buffer to give a final concentration of about 10^{-5} M. The solution was maintained at 37°C. A solution of 325 μl of porcine esterase was added to achieve a concentration of 1.3 U/ml. Aliquots of 300 μl were withdrawn every 15 min for 2 h and combined with 600 μl of 0.01 N HCl in methanol. After centrifugation at $5000 \times g$ for 5 min an aliquot of supernatant was monitored by HPLC. Pseudo first-order rate constants for enzymatic hydrolysis were determined from the slopes of linear plots obtained by reporting the logarithm of residual 7-chlorokynurenic ester against time. All experiments were carried out in triplicate.

2.6. Analytical procedures

HPLC analysis was performed by a Lichrocart RP 18 column (particle size: 5 μm; 250×4 mm I.D.; Merck, Darmstadt, Germany). A gradient program was used: inject sample with mobile phase phosphate buffer (A):methanol (B) (90:10), linearly change mobile phase over 15 min to phosphate buffer (A):methanol (B) (30:70), then return to initial conditions. The flow-rate was set at 1.0 ml/min. Each sample containing the compounds **1**–**4** was filtered prior to injection using a Millex HV13 filter (Waters–Millipore Corporation, Milford, MA, USA) and an aliquot (20 μl) was injected into the HPLC apparatus. The drug was monitored at 344 nm (excitation wavelength) and 398 nm (emission wavelength). The retention times were: 7-chloroKynurenic acid **1,** 6.8 min; ester **2**, 14.4 min; ester **3**, 14.0 min; ester **4**, 13.8 min. The limit of sensitivity was less than 5 μM.

2.7. NMDA-induced seizures in mice

Swiss Webster (30–35 g b.w.) were used. In any experimental session, a maximum of 12 mice were acutely injected i.p. with 7-chlorokynurenic acid **1** or one of its esters **2**–**4**. All compounds **1**–**4** were dissolved in saline (pH adjusted to 7,4 with NaOH), and injected at the fixed dose of 200 mg/Kg. Control mice received saline alone. Fifteen minutes later, all mice were injected with NMDA (200 mg/Kg, i.p., dissolved in saline, pH 7,4) and examined for the following 60 min by an observer who was unaware of the treatment. In control mice, seizures developed through a sequence of paroxysmal scratching, hypermotility and circling, tonic–clonic convulsion, and, occasionally, death. The following semiquantitative scale was used for the examination of the seizure severity: 0, no response; 1, excessive grooming and paroxysmal scratching; 2, mild hypermobility; 3, extensive hypermotility and circling; 4, forepaw clonus and tail hypertonus; 5, generalized tonic/clonic convulsion; 6, 'status epilecticus' and death. The latency (in minutes) for partial seizures (PS) (excessive grooming, scratching or hypermotility) or generalized seizures (GS) (clonic or tonic/clonic convultions) was also determined.

2.8. Extracellular hydrolysis of ester **4** in mixed cultures of mouse cortical cells

Mixed cortical cell cultures containing both neurones and astrocytes were prepared from fetal mice at 14–16 day gestation, as described by Rose et al. (1993). In brief, dissociated cortical cells were plated in 15-mm multiwell vessels (Falcon Primaria, Lincon Park, NY) on a layer of confluent astrocytes, using a plating medium of MEM-Eagle's salts (supplied glutamine free) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, glutamine (2 mM), glucose (21 mM), and $NaHCO_3$ (25 mM). After 3–5 days in vitro (DIV), non-neuronal cell division was halted by a 1–3 day exposure to 10 μM cytosine arabinoside, and cultures shifted to a maintenance medium identical to the plating medium, but lacking fetal bovine serum. Subsequent partial medium replacement was carried out twice a week. Cultures at 13–14 DIV were used.

To examine whether ester prodrug **4** is hydrolyzed into free 7-chlorokynurenic acid (**1**) at the surface of neurones or astrocytes, we have applied compound **4** to mixed cultures of cortical cells. Cultures were incubated for up to 180 min with the above described HEPES-buffered salt solution, and aliquots of the buffer were collected at different times. Culture medium was collected at different times after application of compound **4**. This was filtered and 20 μl samples were injected into the HPLC by a 20 μl loop. We carried out the simultaneous detection of both compounds **1** and **4** by HPLC method described above.

Table 1
Chemical and enzymatic stability of esters **2**–**4**

Compound	$t_{\frac{1}{2}}$ (h)[a]	
	pH 7.4 buffer	Esterase (1.3 U/ml)
2	8.5	1.1
3	5.8	0.5
4	10.2	1.5

[a] $t_{\frac{1}{2}}$ was calculated from the equation: $t_{\frac{1}{2}} = (\ln 0.5)/K^1$, where K^1 is the pseudo-first order constant.

3. Results and discussion

Data concerning chemical and enzymatic stability in pH 7.4 buffer solution and in presence of porcine esterase of ester prodrugs **2**–**4** are reported in Table 1. When we evaluated their chemical stability, the compounds appeared moderately stable in a pH 7.4 buffered solution, however, prodrugs **3** and **4** disappeared more slowly than the ester **2**.

To confirm that the tested prodrugs can be enzymatically hydrolysed, we evaluated their stability in the presence of porcine esterase. The findings indicate that each of the three esters **2**–**4** is capable of being cleaved in vitro by esterase.

Half-life times concerning enzymatic stability were notably lower than those obtained in buffer solution. Moreover we found a good correlation between chemical and enzymatic decomposition rates of the prodrugs examined.

The obtained results suggest that 6-glucosidic linkage (ester **4**) is cleaved easier than 3-glucosidic one (ester **2**).

Table 2 shows the results obtained in in vivo protective activity evaluation of esters **2**–**4** against NMDA induced seizures.

In control mice, injection of NMDA (200 mg/Kg, i.p.) induced generalized tonic/clonic convulsions, causing the death of 13 out of 21 animals. Only two animals exhibited exclusively partial seizures, whereas one animal did not respond to NMDA.

The severity of NMDA-induced seizures was not attenuated in mice pretreated i.p. with 200 mg/Kg of 7-chlorokynurenic acid or ester **3** (see Table 2). In contrast, a pretreatment with either esters **2** or **4** (both at 200 mg/Kg, i.p.) substantially protected against NMDA-induced seizures, with the former showing the highest protective activity. Among 19 mice pretreated with ester **4**, only four developed generalized seizures, and one died. Five of these mice did not show behavioral response to NMDA. In mice pretreated with ester **4** which responded to NMDA, the latency to either partial or generalized seizures was twice as long as in animals preinjected with vehicle alone. All mice ($n = 7$) pretreated with ester **2** showed a behavioral response to NMDA, but only one

Table 2
Seizure severity (SS) score, latency to the onset of partial (PS) or generalized (GS) seizures and mortality in mice treated with NMDA (200 mg/Kg, i.p.), 15 min after the injection of saline, 7-chlorokynurenic acid **1**, esters **2**–**4**[a]

	SS score	Latency PS (min)	Latency GS (min)	Mortality
Saline	5.0 ± 0.5	5.2 ± 1.2	12.0 ± 2.6	13/21
1	4.5 ± 0.8	7.5 ± 1.2	11.0 ± 4.3	3/6
2	2.4 ± 0.5*	7.8 ± 2.1	20.1 ± 7.3	0/7
3	4.2 ± 0.6	9.6 ± 6.9	16.4 ± 3.8	5/11
4	1.8 ± 0.4*	14.0 ± 0.9	23.2 ± 4.4	1/19

[a] Eighteen out of 21 mice preinjected with saline, and four out of six mice preinjected with 7-chlorokynurenic acid **1** exhibited generalized seizures. Only four of 19 mice pretreated with ester **4** and one of seven mice pretreated with ester **2** exibited generalized seizures. Mice's group treated with ester **4** showed the highest number of animals (five of 19) which did not show behavioral response to NMDA (non responders in other groups: one out of 21 in saline-treated mice; 0/6 in 7-chlorokynurenic acid-treated mice; 0/11 in ester **3**-treated mice and 0/7 in ester **2**-treated mice).

* $P<0.01$ (one-way ANOVA+ Fisher PLSD), if compared to saline-treated mice.

developed generalized convulsions and none of them died. In these animals, however, the latency to partial seizures was comparable to that observed in controls (Table 2).

The high in vivo protective activity of systematically administered esters **3** and especially **4** against NMDA-induced seizures and the inefficiency of 7-chlorokynurenic acid **1** under the same experimental conditions, suggests that the glucose moiety could act as a vector, transporting these esters across the blood–brain barrier, beyond which they could be hydrolyzed into parent drug **1**. Since D-glucose and D-galactose enter the brain through the transporter GLUT-1 (Fuglsang et al., 1986), we suppose that esters **2** and **4** could be transported across the blood-brain barrier by the same carrier. As this carrier has a lower affinity for D-galactose than for D-glucose (Fuglsang et al., 1986) it is likely that ester **3** is inactive against NMDA-induced seizures because it is not efficiently transported. Accordingly, other authors (Rodriguez et al. 1989) found that galactosyl enkephalin analogues were much more potent than glucosyl analogues in inducing analgesia when injected intracerebroventricularly, suggesting that galactoside analogues are not transported by GLUT-1 out of the brain (Polt et al., 1994).

To examine whether ester **4**, which shows the better pharmacological profile, is hydrolyzed to generate 7-chlorokynurenic acid **1** at the target site, we have used mixed cultures of neurons and glial cells, a model that roughly reproduces the cellular milieu encountered by the prodrug after it cross the blood-brain barrier. Ester **4** was applied to cultures incubated in a salt-balanced buffer solution, to avoid any interference by enzymes or enzyme inhibitors present in the serum. The amount of 7-chlorokynurenic acid **1** applied to the cultures (173 ± 18 μM, $n=3$) did not change in the extracellular medium after 3 h of incubation (170 ± 10 μM,), indicating that free 7-chlorokynurenic acid **1** is not taken up by neurons or astrocytes and is not metabolized at the cell

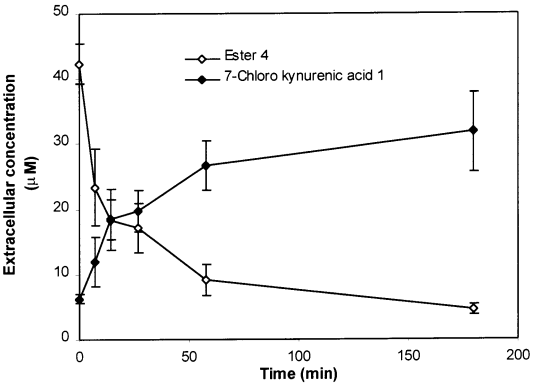


Fig. 2. Simultaneous detection of ester **4** and free 7-Cl-Kynurenic acid in the extracellular medium of cultured cells incubated at different times with ester **4**. Values are means ± SEM of six individual determinations. 'Time 0' values were determined by diluting the prodrug with the same amount of buffer present in the culture dishes. Note that a small amount of free 7-Cl-Kynurenic acid **1** is already present at time 0, which may reflect some contamination with free 7-Cl-Kynurenic acid **1** originating from the chemical synthesis.

surface. Application of ester **4** was instead followed by a rapid decline in their extracellular concentration, with a $t_{1/2}$ of about 10 min (see Fig. 2).This effect was accompanied by a parallel rise in extracellular concentration of free 7-chlorokynurenic acid 1, with the two curves crossing after 15 min about.

The in vivo and in vitro results obtained in this study indicate that ester **4** is an excellent prodrug for potential application in the experimental therapy of epilepsy, as well as of neurodegenerative disorders of excitotoxic origin. Further studies are in progress to clarify the mechanism of its pharmacological activity.

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